Seq. Box

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NO.	U.S. APPLICATION NO.
206579	Unassigned 09/646651

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/07722	30 November 1998	13 March 1998

TITLE OF INVENTION
METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

APPLICANT(S) FOR DO/EO/US
Kiesewetter et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) now rather than delay examination until the expiration of the applicable time limit set forth in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendment to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☒ A copy or translation of the Amendments made by the Applicant during PCT Chapter II, which are attached as Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 17 below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment. ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ A verified small entity statement.

17. ☒ Other items or information:
Nucleotide and/or Amino Acid Sequence Submission (Computer Readable Copy and Paper Copy (2 pages))

428 Rec'd PCT/PTO 13 SEP 2000

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
Unassigned 09/646651	PCT/EP98/07722	206579

18. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO ..... $ 840.00	$840.00	
International preliminary examination fee paid to USPTO..... $ 670.00	$	
No international preliminary examination fee paid to USPTO, but international search fee paid to USPTO ..... $ 690.00	$	
Neither international preliminary examination fee nor international search fee paid to USPTO ..... $ 970.00	$	
International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1) to (4) ..... $ 96.00	$	
ENTER APPROPRIATE BASIC FEE AMOUNT=	$840.00	
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date	$0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	11 -20=	0	x $ 18.00	$0.00	
Independent Claims	1 - 3 =	0	x $ 78.00	$0.00	
☐ Multiple Dependent Claim(s) (if applicable)			+$260.00	$0.00	

TOTAL OF ABOVE CALCULATIONS=	$840.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed.	$0.00	
SUBTOTAL=	$840.00	
Processing fee of **$130.00** for furnishing English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date.	$0.00	
TOTAL NATIONAL FEE=	$840.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. **$40.00** per property +	$0.00	
TOTAL FEE ENCLOSED=	$840.00	
Amount to be: refunded	$	
charged:	$	

a. ☒ A check in the amount of $840.00 to cover the above fee is enclosed.

b. ☐ Please charge Deposit Account No. 12-1216 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1216. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

LEYDIG, VOIT & MAYER, LTD.
Two Prudential Plaza, Suite 4900
180 North Stetson
Chicago, Illinois 60601-6780
(312) 616-5600 (telephone)
(312) 616-5700 (facsimile)

Salim A. Hasan, Registration No. 38,175
One of the Attorneys for Applicant(s)

The PTO did not receive the following listed item(s) NO Check

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
Unassigned 09/646651	PCT/EP98/07722	206579

CERTIFICATION UNDER 37 C.F.R. § 1.10

"Express Mail" Label Number: EL643537230US

Date of Deposit: September 13, 2000

I hereby certify that this express request to begin national examination procedures under 35 U.S.C. § 371(f) of the International Patent Application referenced above, including all of the items listed thereon as enclosures, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner for Patents, Attention: DO/EO/US, Washington, D.C. 20231.

Rick D. Madsen — Printed Name of Person Signing:

Rick D. Madsen — Signature

#9

PATENT
Attorney Docket No. 206579

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Kiesewetter et al.

Application No.: 09/646,651

Group Art Unit: Unassigned

Examiner: Unassigned

Filed: September 13, 2000

For: METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

SUBMISSION OF SEQUENCE LISTING

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In accordance with the requirements of 37 CFR 1.821-1.825, a sequence listing is being submitted as part of the patent application. The sequence listing is in the form of both a paper copy and a computer readable copy on a computer diskette. The undersigned hereby verifies that the content of the paper copy and the computer readable copy, as concurrently being submitted, are the same. The sequence listing filed on September 13, 2000 has been amended to correct errors as pointed out by the "Notification to Comply With Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Listing Disclosures" dated May 10, 2001. Thus, the sequence listing submitted herewith does not include any new matter and Applicants hereby request that the sequence listing submitted herewith be substituted for the one submitted on September 13, 2000.

Respectfully submitted,

Salim A. Hasan, Reg. No. 38,175
One of the Attorneys for Applicant(s)
LEYDIG, VOIT & MAYER, LTD.
Two Prudential Plaza, Suite 4900
180 North Stetson
Chicago, Illinois 60601-6780
(312) 616-5600 (telephone)
(312) 616-5700 (facsimile)

Date: May 16, 2001

#3

PATENT
Attorney Docket No. 206579

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Kiesewetter et al.

Group Art Unit: Unassigned

Application No. 09/646,651

Examiner: Unassigned

Filed: September 13, 2000

For: METAL-CONTAINING
RIBONUCLEOTIDE POLYPEPTIDES

SUPPLEMENTAL PRELIMINARY AMENDMENT

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

AMENDMENTS

In the Specification

Please amend the specification as follows:

Page 2, after "KDQGTIDKFQNLDANQDEQVSFKEFVVLVTDVLITA HDNIHKE-COOH" insert -- [SEQ ID NO:1] --.

CERTIFICATE OF MAILING

I hereby certify that this **SUPPLEMENTAL PRELIMINARY AMENDMENT** (along with any documents referred to as attached or enclosed) is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on the date shown below.

Date: October 4, 2000 Kathleen M. Lamb

Page 3, after "GGAAAAUNNNNNUNAUAUGN$_{1-6}$CUNNNUUUNNNNNN AAAAAN$_{0-1}$UANAAACAUN$_{0-5}$CUUNAGN$_{0-13}$AGAAAUN$_{0-16}$UUAGCAG" insert -- [SEQ ID NO:2] --.

Page 3, after "AAAAAAAAGGUUUUCAUGCGUGCUCACAGAUCAG CUCUUUCUGGAUUGAAAAGCUAAGCACAGAACAUGGGAAAAUUCCUUU CAUAUGGCUGUGUUUACAAACAAAAAGUAUAAACAUCUUGAGCAAACA GAAAUGGUGAGGAAAACUUUGUUAGCAGAUUAG" insert -- [SEQ ID NO:3] --.

Page 3, after "UUACAGCUCUUCUGUUUAUAAGUUAUUCAAUACCA AAUUAGUAGUUUGUAUGUUAUAAAUUUGUAGGAAAAUAAUUAUAUAU GCUUACUUUGUACAUAAAAAUAAAAACAUGACUUCUUUAGACACUCCU UCAUUAGAAAUAAAAUAAAAUAAACUAUUAGCAGUUUGACUUCAUGU UCUGUCUGUAGGUCAUGGAAUCCUGUCCUUACAAUAUUUAUUGAUUGU GAAAAUAUCAGUAAAUAAGCAAUUGAAUAUGUUUACCUUUUCUUCUAG UCACUAUGUUCUUAGAGUUAUGACA" insert -- [SEQ ID NO:4] --.

Page 4, after "(a1)" insert -- [SEQ ID NO:3] -- and after "(a2)" insert -- [SEQ ID NO:4] --.

Page 26, after "ARNA I" insert -- [SEQ ID NO:2] -- and after "ARNA VI" insert -- [SEQ ID NO:3] --.

In the Claims

Please delete claims 1-11 and add claims as follows:

12. A metal-containing ribonucleotide protein (RPN) containing a protein from the family of S100 proteins, an RNA and copper as metal ion in the form of a ternary complex, characterized in that the RNA of the ternary complex has the following sequences:

(a1) ARNA I

Klon-3a (ARNA I)

AAAAAAAAGGUUUUCAUGCGUGCUCACAGAUCAGCUCUUUCUGG
AUUGAAAAGCUAAGCACAGAACAUGGGAAAAUUCCUUUCAUAUGGCUG
UGUUUACAAACAAAAAGUAUAAACAUCUUGAGCAAACAGAAAUGGUGA
GGAAAACUUUGUUAGCAGAUUAG [SEQ ID NO:3]

or

(a2) ARNA VI

Klon-P10 (ARNA VI)

UUACAGCUCUUCUGUUUAUAAGUUAUUCAAUACCAAAUUAGUAG
UUUGUAUGUUAUAAAUUUGUAGGAAAAUAAUUAUAUAUGCUUACUUU
GUACAUAAAAAUAAAAACAUGACUUCUUUAGACACUCCUUCAUUAGAA
AUAAAAUAAAAUAAACUAUUAGCAGUUUGACUUCAUGUUCUGUCUGUA
GGUCAUGGAAUCCUGUCCUUACAAUAUUUAUUGAUUGUGAAAAUAUCA
GUAAAUAAGCAAUUGAAUAUGUUUACCUUUUCUUCUAGUCACUAUGUU
CUUAGAGUUAUGACA [SEQ ID NO:4]

13. A process for producing a metal-containing ribonucleotide protein (RPN) according to claim 1, characterized in that leucocytes or inflammation tissue is homogenized or leucocytes are cultivated and the resulting RNP is recovered from the homogenates or from the supernatant of the culture solution by standard methods.

14. A use of the metal-containing ribonucleotide protein (RPN) according to claim 1 and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

REMARKS

The Present Invention

The present invention is directed to metal containing ribonucleotide polypeptides (RNPs) (claim 12), and a process (claim 13) and use (Claim 14) thereof.

Discussion of Amendments

The specification and claims have been amended to provide sequence listing information. As such, no new matter has been added. A complete set of the amended claims is attached hereto for the convenience of the Examiner.

Conclusion

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

Kathryn M. Lumb, Reg. No. 46,885
One of the Attorneys for Applicants
LEYDIG, VOIT & MAYER, LTD.
Two Prudential Plaza, Suite 4900
180 North Stetson
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(312) 616-5600 (telephone)
(312) 616-5700 (facsimile)

Date: October 4, 2000

206579SPA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Kiesewetter et al.

Group Art Unit: Unassigned

Application No. 09/646,651

Examiner: Unassigned

Filed: September 13, 2000

For: METAL-CONTAINING
RIBONUCLEOTIDE POLYPEPTIDES

PENDING CLAIMS AS OF OCTOBER 4, 2000

12. A metal-containing ribonucleotide protein (RPN) containing a protein from the family of S100 proteins, an RNA and copper as metal ion in the form of a ternary complex, characterized in that the RNA of the ternary complex has the following sequences:

(a1) ARNA I

Klon-3a (ARNA I)

AAAAAAAAGGUUUUCAUGCGUGCUCACAGAUCAGCUCUUUCUGG AUUGAAAAGCUAAGCACAGAACAUGGGAAAAUUCCUUUCAUAUGGCUG UGUUUACAAACAAAAAGUAUAAACAUCUUGAGCAAACAGAAAUGGUGA GGAAAACUUUGUUAGCAGAUUAG [SEQ ID NO:3]

or

(a2) ARNA VI

Klon-P10 (ARNA VI)

UUACAGCUCUUCUGUUUAUAAGUUAUUCAAUACCAAAUUAGUAG
UUUGUAUGUUAUAAAUUUGUAGGAAAAUAAUUAUAUAUGCUUACUUU
GUACAUAAAAAUAAAAACAUGACUUCUUUAGACACUCCUUCAUUAGAA
AUAAAAUAAAAUAAACUAUUAGCAGUUUGACUUCAUGUUCUGUCUGUA
GGUCAUGGAAUCCUGUCCUUACAAUAUUUAUUGAUUGUGAAAAUAUCA
GUAAAUAAGCAAUUGAAUAUGUUUACCUUUUCUUCUAGUCACUAUGUU
CUUAGAGUUAUGACA [SEQ ID NO:4]

13. A process for producing a metal-containing ribonucleotide protein (RPN) according to claim 1, characterized in that leucocytes or inflammation tissue is homogenized or leucocytes are cultivated and the resulting RNP is recovered from the homogenates or from the supernatant of the culture solution by standard methods.

14. A use of the metal-containing ribonucleotide protein (RPN) according to claim 1 and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

SEQUENCE LISTING

<110> Kiesewetter, Stefan
Kuhn, Eckehard
Koch-Pelster, Brigitte
Brunner, Herwig

<120> METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

<130> 206579

<140> PCT/EP98/07722
<141> 1998-11-30

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1 5 10 15

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Lys Tyr Leu Ile Thr Lys Glu Leu Pro Asn Thr Leu Lys Asn Thr Lys
35 40 45

Asp Gln Gly Thr Ile Asp Lys Ile Phe Tyr Asn Leu Asp Ala Asn Gln
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agacacuccu ucauuagaaa uaaaauaaaa uaaacuauua gcaguuugac uucauguucu 180

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uaagcaauug aauauguuua ccuuuucuuc uagucacuau guucuuagag uuaugaca 298

PATENT
Attorney Docket No. 206579

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Kiesewetter et al.

Group Art Unit: Unassigned

Serial No. Unassigned

Examiner: Unassigned

Filed: September 13, 2000

For: METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

AMENDMENTS

Please amend the claims as follows:

4. (Amended) Antibody directed towards the ribonucleotide proteins according to [one of claims] claim 1 [to 3].

7. (Amended) Use of the ribonucleotide proteins according to [one of claims] claim 1 [to 3] and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

Please add claims 8-11 as follows:

8. Antibody directed towards the ribonucleotide proteins according to claim 2.

9. Antibody directed towards the ribonucleotide proteins according to claim 3.

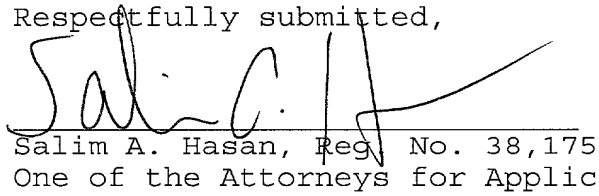
10. Use of the ribonucleotide proteins according to claim 2 and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

11. Use of the ribonucleotide proteins according to claim 3 and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

REMARKS

The claims have been amended in order to place the claims in a format more suitable to U.S. patent practice. No new matter has been added by way of these amendments. The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



Salim A. Hasan, Reg. No. 38,175
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Chicago, Illinois 60601-6780
(312) 616-5600 (telephone)
(312) 616-5700 (facsimile)

Date: September 13, 2000

Translation of basic PCT application (31 pages in total)

Metal-containing ribonucleotide polypeptides

The present invention relates to metal-containing ribonucleotide polypeptides (RNP) and processes for their preparation, their use and medicaments containing ribonucleotide polypeptides or their molecular-biological equivalent structures and/or parts and/or derivatives.

Tissue homeostasis of the body, its organs and tissue depends on the regulation mechanisms of angiogenesis. This influences both tissue repair and wound healing, formation of new tissue in embryogenesis and the reproductive cycles and growth, retrogression and destruction of tumours, transplants and vascularised and non-vascularised tissues.

Hitherto, no non-mitogenic mediators have yet been found, with which influence of tissue homeostasis is possible, that is induction and regulation of vascular growth.

The object of the present invention is therefore to provide a non-mitogenic mediator of tissue homeostasis, with which primarily tissue repair, wound healing, angiogenesis and neovascularisation may be influenced. A further object of the invention is the provision of a process for producing the non-mitogenic mediators and a medicament containing this non-mitogenic mediator.

These aims are achieved by the objects of the patent claims.

It has been found by the inventors that there are non-mitogenic cellular mediators based on nucleic acid of defined sequence, which may cause specifically the formation of blood vessels in vivo and in vitro and represent biologically specific, naturally acting non-mitogenic mediators of angiogenesis or the directional growth of blood vessel branches.

The new class of cellular morphogens for endothelial cells proven by the inventors exists in the form of a bioactive metal ribonucleotide peptide (RNP). This is called angiotropin.

The structure of angiotropin may be assigned to the ribonucleotide proteins (RNP). It consist of a protein part (ARP .= Angiotropin Related Protein) and an RNA part (ARNA = Angiotropin RNA). Cu(II) is essential for formation of the complex of ARP and ARNA. In addition to the copper ion, angiotropin contains a Ca(II) ion. Mg(II) ions are also useful for the diverse biological and biochemical functions of angiotropin.

The protein part (ARP) consists of a protein which may be assigned to the family of S100 proteins (Dell'Angelica et al., Journal of Biological Chemistry, Volume 269, No. 46, page 28929-28936 (1994)) and is preferably 91 amino acids long. The primary structure of this preferred ARP is as follows:

Ca^{2+}

NH_2-TKLEDHLEGIINIFHQYSVRLGHYDTLIKRELKQLITKELPNTLKNT

Ca^{2+} Zn^{2+}

KDQGTIDKIFQNLDANQDEQVSFKEFVVLVTDVLITAHDNIHKE-COOH

It has two EF-hand motives and furthermore a binding site for zinc(II) ions. The dissociation constants K_D of the metal ion complexes are 10.0 µM for Ca(II) ions or 0.1 µM for zinc(II) ions and 1.0 µM for copper(II) ions.

In this context, it should be mentioned that the protein part of the invention may be modified according to conventional processes known in the expert field without the biological activity being lost. These modifications include exchanges, insertions or deletions of amino acids, which modify the structure of the protein part, its biological activity being essentially maintained. The exchanges preferably include "conservative" exchanges of amino acid residues, that is exchanges for biologically similar residues, for example the substitution of a hydrophobic residue (for example isoleucine, valine, leucine, methionine) for a different hydrophobic residue, or the substitution of a polar residue for a different polar residue (for example arginine for lysine, glutamic acid for asparaginic acid etc.). Deletions may lead to the production of molecules which have a

significantly smaller size, that is those lacking, for example amino acids at the N or C terminus.

The RNA part (ARNA) has the following consensus sequence:

$$\text{GGAAAAUNNNNNUNAUAUGN}_{1\text{-}6}\text{CUNNNUUUNNNNNNAAAAAN}_{0\text{-}1}$$
$$\text{UANAAACAUN}_{0\text{-}5}\text{CUUNAGN}_{0\text{-}13}\text{AGAAAUN}_{0\text{-}16}\text{UUAGCAG}$$

wherein "N" is G, A, U or C,
or the complementary consensus sequence thereof.

According to the invention the ARNAs which can be used are further defined as follows:

(A1) ARNA I

Klon-3a (ARNA I)
AAAAAAAAGGUUUUCAUGCGUGCUCACAGAUCAGCUCUUUCUGGAUUGAAAAGCU
AAGCACAGAACAUGGGAAAAUUCCUUUCAUAUGGCUGUGUUUACAAACAAAAAGU
AUAAACAUCUUGAGCAAACAGAAAUGGUGAGGAAAACUUUGUUAGCAGAUUAG

or an RNA which is different therefrom by one or more base pairs or a fragment thereof,

or

(A2) ARNA VI

Klon-P10 (ARNA VI)
UUACAGCUCUUCUGUUUAUAAGUUAUUCAAUACCAAAUUAGUAGUUUGUAUGUUA
UAAAUUUGUAGGAAAAUAAUUAUAUAUGCUUACUUUGUACAUAAAAAAUAAAAACAU
GACUUCUUUAGACACUCCUUCAUUAGAAAUAAAAUAAAAUAAACUAUUAGCAGUUU
GACUUCAUGUUCUGUCUGUAGGUCAUGGAAUCCUGUCCUUACAAUAUUUAUUGAU
UGUGAAAAUAUCAGUAAAUAAGCAAUUGAAUAUGUUUACCUUUUCUUCUAGUCAC
UAUGUUCUUAGAGUUAUGACA

or an RNA which is different therefrom by one or more base pairs or a fragment thereof.

The nucleic acid molecules defined under (a1) and (a2) also include nucleic acid molecules which are different with respect to the above-mentioned sequence due to deletion(s), insertion(s), exchange(s) or other modifications known in the state of the art, without the biological activity being lost. The term "nucleic acid fragment" should include a cutout or segment of the original nucleic acid molecule. Processes for producing the above changes in the nucleic acid sequence are known to the expert and described in standard works on molecular biology, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989).

The ARNAs mentioned also include molecular-biological equivalent structures, that is structures in which individual bases or amino acids are exchanged. They also include therewith hybridising nucleic acids (preferably under stringent conditions, such as 20°C below the melting point of RNA) or nucleic acids related via the degenerated genetic code.

The RNPs of the invention are characterised by the following properties:

- cell-selective morphogenic effect in vitro on isolated, primary and/or cloned blood capillary endothelial cells in culture for non-mitogenic induction of change of the cell phenotype from the confluent state, for non-mitogenic change of the spatio-temporal supracellular organisation of cells to form three-dimensional organoid, capillary-similar structures in culture;
- specific chemotropic effect on blood vessels in vivo,
- induction of directional growth of blood vessels in vivo,
- induction of neovascularisation of tissues by directed inward growth of blood vessels.

In the RNPs of the invention, the protein part is bound to the RNA part by interactions.

The object of the invention is also a process for producing and recovering the bioactive RNPs, characterised in that the cells, for example the leucocytes or inflammation tissue is homogenised or leucocytes are cultivated and the resulting RNPs are recovered from the homogenates or from the supernatants of the culture solution by standard methods. For example angiotropin may be isolated from the supernatants of serum-free mass cell cultures of concanavaline A-activated porcine blood leucocytes and leucocytes from ischaemic/infarcted heart muscle cells and purified to homogeneity.

The process for producing and recovering the bioactive RNP morphogens of cells or leucocytes and inflammation tissue is characterised in that cells or leucocytes of the reticulo-endothelial system of leucocytes and inflammation tissue, are cultivated and the resulting RNP morphogens are recovered from the homogenates or from the supernatant culture solution.

In principle, it is also possible to work up the cells, for example leucocytes on mediators directly without culture.

Culture of the cells (leucocytes) may be carried out in principle in any medium maintaining the cells (leucocytes).

For the culture of cells, such as leucocytes, in most cases serum, for example calf serum or horse serum, is added to the culture media for a planned duration of culture over 1 hour, since the serum constituents are favourable for the maintenance of the vital functions of the cells. If however the serum-containing culture solution is to be worked up on proteins (mediators), which are produced by the culture, recovery of the product proteins present mostly only in low concentrations creates considerable difficulties due to the number of foreign proteins originating from the serum. In addition, it is thus not

possible to establish with certainty whether a certain mediator is of humoral or cellular origin and from which species it comes; that is whether it is a mediator of the species, the cells of which have been cultivated, or the species, from which the serum used (mostly heterologous) comes.

The fully synthetic cell culture medium preferably used according to the invention contains the conventional material groups, such as salts, sugars, amino acid, nucleosides and nucleoside bases, vitamins, vitaminoids, coenzymes and steroids in aqueous solution. It is characterised in that it additionally contains one material or a mixture of several materials, which have proved to be particularly valuable for the vitality and the growth of the leucocytes and their ability for mediator production. These materials include unsaturated fatty acids, flavonoids, ubiquinones, vitamin C and mevalolactone.

The cell culture medium is used for longer-lasting cell or leucocyte culture, preferably without adding serum. Instead, it has at least one defined protein, which is highly pure, molecularly uniform serum albumin in a particularly preferred embodiment.

In further preferred embodiments, the fully synthetic serum-free cell culture medium used according to the invention may contain still further compounds which are favourable for the culture of leucocytes from the material classes of polyhydroxy compounds and sugars, amino acids, nucleosides, anionic compounds and/or vitamins, the use of which is not conventional in the known culture media. The constituents of the medium used according to the invention are adjusted to one another with regard to their quantitative proportions so that the concentration of the components in the medium is largely matched to the natural concentration ranges of the plasma; see Ciba-Geigy AG (Publisher) (1969) in Documenta Geigy, Wissenschaftliche Tabellen [Scientific Tables], 7th Edition Geigy S.A., Basle.

The cell culture medium is preferably free of surfactants, heavy metal salts and dyestuffs which damage cells and may disturb the recovery of the required cell products from the culture solution.

The cell culture medium having the composition indicated in Table 1 below is particularly preferred for the culture of the leucocytes in the process of the invention.

Water having ATM-1 quality is used for producing the medium; see ASTM D-1193-70 Standard Specification for Reagent Water 1970; Annual Book of ASTM Standards, Easton Maryland, ASTM 1970. Furthermore, it is freed of possible endotoxin contaminations due to ultrafiltration on surfactant-free membranes having the exclusion limit of 10,000 Dalton. The final medium is filter-sterilised on surfactant-free membranes having ≤ 0.2 µm pore size.

Table 1

No.	Component	mole/litre
1	KCl	5.0 m
2	KH_2PO_4	0.2 m
3	NaCl	120.0 m
4	Na_2HPO_4	0.8 m
5	Na_2SO_4	0.8 m
6	L-Ascorbic acid	0.2 m
7	Choline chloride	50.0 µ
8	2-Deoxy-D-ribose	5.0 µ
9	D-Galactose	0.5 m
10	D-Glucose	5.0 m
11	D-Glucurono-Y- lactone	0.1 m
12	Glycerol	50.0 µ
13	myo-Inositol	0.5 m
14	Na acetate	0.2 m
15	Na citrate	50.0 µ
16	Na pyruvate	0.1 m
17	D-Ribose	20.0 µ
18	Succinic acid	0.1 m
19	Xylitol	10.0 µ
20	D-Xylose	20.0 µ
21	$CaCl_2$	2.0 m
22	$MgCl_2$	1.0 m
23	$NaHCO_3$	10.0 m
24	Human serum albumin	7.7 µ
25	Penicillin	1.0 µ

No.	Component	mole/litre
26	Streptomycin	2.0 μ
27	L-Glutamine	1.0 m
28	L-Alanine	0.2 m
29	L-Asparagine	0.1 m
30	L-Asparaginic acid	0.1 m
31	L-Glutamic acid	0.1 m
32	Glycine	0.2 m
33	L-Proline	0.1 m
34	2L-Serine	0.1 m
35	L-Arginine	0.1 m
36	4-Aminobenzoic acid	2.0 μ
37	L-Cysteine	0.2 m
38	L-Histidine	0.1 m
39	L-Hydroxyproline	10.0 μ
40	L-Isoleucine	0.2 m
41	L-Leucine	0.2 m
42	L-Lysine HCl	0.2 m
43	L-Methionine	0.1 m
44	L-Ornithine	50.0 μ
45	L-Phenylalanine	0.1 m
46	Sarcosine	50.0 μ
47	Taurine	0.1 m
48	L-Threonine	0.2 m
49	L-Tryptophane	50.0 μ
50	L-Tyrosine	0.1 m
51	L-Valine	0.2 m
52	Glutathione reduced	3.0 μ
53	Carnosine	5.0 μ

No.	Component	mole/litre
54	Mevalolactone	5.0 μ
55	Adenine	50.0 μ
56	Adenosine	50.0 μ
57	Citidine	50.0 μ
58	Guanine	5.0 μ
59	Guanosine	20.5 μ
60	Hypoxanthine	5.0 μ
61	5-Methylcytosine	5.0 μ
62	Thymidine	20.0 μ
63	Thymine	5.0 μ
64	Uracil	5.0 μ
65	Uridine	20.0 μ
66	Xanthine	5.0 μ
67	Biotin	1.0 μ
68	C-Ca pantothenate	5.0
69	Ergocalciferol	0.5 μ
70	D, L-Carnitine	50.0 μ
71	Folic acid	5.0 μ
72	D, L-α-lipoic acid	2.0 μ
73	Menadione	0.2 μ
74	Nicotinamide	20.0 μ
75	Pyridoxal HCl	5.0 μ
76	Pyridoxine HCl	2.0 μ
77	Riboflavin	1.0 μ
78	Rutin	5.0 μ
79	Thiamine HCl	5.0 μ
80	D, L-α-tocopheryl acetate	1.0 μ
81	Vitamin A acetate	1.0 μ

No.	Component	mole/litre
82	Vitamin K	0.2 µ
83	Vitamin B^1	0.5 µ
84	Vitamin U^{12}	1.0 µ
85	Cholesterol	1.0 µ
86	Coenzyme Q_{10}	0.1 µ
87	Linolic acid	1.0 µ
88	Linolenic acid	5.0 µ
89	Oleic acid	5.0 µ
90	Ethanol	1.0 m
91	pH 7.10	--
92	Concanavaline A	50.0 n

Depending on the type of products required, either mixed cell or leucocyte populations or individual cell or leucocyte types are cultivated. The production and culture of the cells or leucocytes must be effected under sterile conditions. Culture is carried out for a sufficiently long time to obtain a satisfactory mediator yield. A period of about 10 to 50 hours has proved to be suitable for this. With shorter times, the mediator yield is too low, so that the process is uneconomical. On the other hand, for a culture period over about 50 hours, the medium is spent and the cells start to die off, so that an increase in yield can no longer be expected.

Culture of the cells or leucocytes is carried out at a temperature of about 30 to 42°C, preferably about 37°C. At lower temperatures the culture process is unsatisfactory, whereas at temperatures above 42°C the leucocytes are damaged.

Culture is carried out at a concentration of about 10^6 to 5 x 10^8 cells/ml, preferably up to 10^7 to 10^8 cells/ml. At lower cell concentrations, the yield per unit volume of culture solution is too low. The process becomes uneconomical due to large culture volumes. At

cell concentration above 5 x 10^8 cells/ml, there is very rapid reduction of nutrients in the medium.

Culture may be carried out in the atmosphere. An increased carbon dioxide partial pressure is preferably maintained above the culture and may reach to about 10 volume %, in particular to about 2 volume %. The oxygen supply to the culture is of considerable importance. It may be ensured, for example by introducing air. In order to avoid contamination of the culture, the air supplied is preferably sterilised and decontaminated by heat, that is freed of endotoxins and other organic constituents. The solution may be stirred or shaken during culture. Con A is preferably used as cell stimulant.

To complete the culture, the cells or leucocytes are centrifuged off from the culture solution, which is then worked up on the resulting angiotropins. In order to avoid damage to the cells and hence contamination of the culture solution due to cell constituents, the culture is centrifuged at relatively low acceleration, that is about 300 to 400 x g. After separating off the greater portion of the cells from the supernatant, the latter is advantageously centrifuged again at higher acceleration to remove residual suspended particles. The leucocytes separated off may either be cultivated again, cryopreserved or passed to a different use.

Apart from culture of leucocytes, the bioactive RNP morphogens of the invention may also be recovered from inflammation tissue. They are produced there due to the accumulation of leucocytes as a result of the inflammation process triggered by tissue damage. The inflammation tissue may be recovered in conventional manner and used for the preparation of RNP. The inflammation tissue is thus homogenised in buffer solution and the soluble constituents (exudate) are separated from the insoluble structural constituents of the tissue.

Inflamed, infarcted heart muscle tissue, which is formed by ligation of the left front descending branch of the left coronary artery by means of a transfemoral catheter technology for 24 hours, is preferably used. The inflamed heart muscle part containing leucocytes is separated off at 0 to 4°C from non-infarcted, healthy tissue.

The working up of a very large culture solution volume is required for the isolation and recovery of the bioactive RNP of the invention. It is therefore necessary for practical reasons at the start of the purification process to carry out as effective as possible reduction of the volume to be treated. The culture solution contains the mixture of the constituents of the medium in addition to the small quantities of substances produced, including mainly proteins. Separation of the proteins produced from the constituents of the medium and at the same time from the large volume of aqueous solution, is therefore advantageously carried out in the first step of purification. This may be effected by selective salting-out of the proteins from the culture solution, which is achieved, for example by adding a sulphate or phosphate. Precipitation of the proteins using the example of salting-out by adding ammonium sulphate to the culture solution is described below.

A large portion of the proteins produced, together with optionally present serum albumin, is precipitated by saturation of the culture solution with ammonium sulphate. After separating off the substance precipitate, for example by centrifuging, the latter may be separated into its individual components in the manner described below and the bioactive RNP present recovered. The supernatant obtained also contains the part of the substances which are soluble in saturated ammonium sulphate solution, also including part of the bioactive RNP, in addition tot he soluble constituents of the medium. The supernatant is concentrated and the substances present are recovered therefrom in the manner below. If the protein-containing culture solution is treated to saturation with ammonium sulphate, the larger part of the concomitant proteins is precipitated. A protein mixture, which consists of a number of different proteins and the separation of which into the individual

components is consequently laborious, is obtained in this manner. In a preferred embodiment of the process of the invention, the protein mixture present in the culture solution is therefore already separated into several fractions in the precipitation stage. This separation into several protein fractions is possible, since the individual proteins are precipitated at different ammonium sulphate concentrations. The culture solution in the process of the invention is therefore preferably treated in stages with ammonium sulphate up to certain degrees of saturation, wherein in each fraction the part of the proteins precipitates, the solubility product of which lies below the particular degree of saturation. In the process of the invention, coarse separation into groups of proteins may be achieved even during precipitation by suitable selection of saturation limits of the individual fractions.

By way of example, the culture solution is initially treated with ammonium sulphate up to a saturation of 35 %. The protein precipitate obtained is separated off. The degree of saturation of the supernatant solution is then increased to 45 %. A protein precipitate is formed again, which is separated off. The supernatant solution is then set to a degree of saturation of 90 %. The protein precipitate thus obtained is likewise separated off. The supernatant solution of this precipitate is concentrated, for example by water-removing dialysis or ultrafiltration.

Salt precipitation of the proteins is carried out, as is the subsequent purification, preferably at a temperature of about 0 to 10°C, in particular about 0 to 4°C. The solutions used for purification have a pH value between 5 and 9, in particular between 6 and 8. In order to achieve pH constancy for the solution, a strong buffer, for example 0.1 mole/litre phosphate buffer, is preferably added before salt precipitation. To maintain the redox potential of the proteins, cysteine is preferably added to the solutions in a quantity of 0.001 mole/litre. Sterile conditions for protein purification are not necessary.

The proteins obtained during salt precipitation may be passed directly to purification and separation described below after dissolving in a medium which does not damage proteins. The supernatant of the last precipitation step is concentrated, for example by water-removing dialysis or ultrafiltration. All compounds having a molecular weight of more than about 300 to 500 Dalton, that is also the proteins and peptides of this fraction, are thus obtained quantitatively as retentate.

The protein fractions obtained in the stage described above contain the bioactive RNP of the invention mixed with numerous foreign proteins (other secreted proteins optionally serum albumin and optionally CON). The foreign proteins exist in by far the predominant quantity in the mixtures. The bioactive RNP has to be enriched by a series of purification steps and freed of the foreign proteins until they no longer disturb their molecular biological specificity. The bioactive RNP themselves are likewise a material class, which is divided into its individual, specifically acting individual parts.

Purification processes for albuminous substances (proteins) and other natural materials generally consist of a sequence of combined separation processes, which utilise differences in molecular size, charge, shape, structural stability and molecular surface condition between the required active ingredient and the concomitant foreign materials for separation. Accordingly, numerous combinations of the widest variety of separation processes may be worked out for purification of a protein. For managing properties, technical feasibility, ability for automation and economic viability of a purification process and for the quality of the required natural product, therefore not only is the type of separation steps used important, but in particular their optimised arrangement and their useful combination in a purification sequence within the framework of the structural stability and the other structural parameters of the required active ingredient. This also means that even the use of the same or similar separation principles (for example molecular sieve filtration, dialysis, ion-exchange adsorption, etc.), but in different combination, may be crucial for the manageability and economic viability of the

purification process. In certain cases, the use or omission of a single technology (for example hydroxylapatite chromatography, zone precipitation chromatography, etc.) is of decisive significance at a certain point of the purification sequence, or within a limited part sequence, for the quality of the required active ingredient and for the manageability and the economic viability of its purification process. These general contexts and basic principles of natural material purification are clearly shown, for example by the generally known fact that in an economically sensible and technically manageable natural material purification process, a column chromatography purification step or a lyophilisation step is not useful, before the total output volume or the output concentration of the concomitant foreign constituents of the active ingredient raw extract has not been reduced to at least 1/500 to 1/1,000 by other process steps.

A plurality of purification steps known individually per se in biochemistry is offered for purification of the individual protein fractions. Examples of such purification steps are: preparative and analytical molecular sieve filtration, anion and cation exchange chromatography or one-pot adsorption processes, chromatography on hydroxylapatite, zone precipitation chromatography and circulation or cascade molecular sieve filtration.

A considerable quantity of concomitant protein may be separated off from the bioactive RNP even by single implementation of one of the said purification processes. However, the substances present in the fractions often adhere very strongly to one another in spite of their different molecular weight. They are often separated incompletely according to their molecular weight, for example in molecular sieve filtration by the existence of non-ideal equilibria for protein polyelectrolytes. It is therefore advisable to carry out at least two of the said separating processes one after another. The protein fractions containing the bioactive RNP activity are preferably subjected to at least three of the said purification steps one after another.

All combinations of the separating steps mentioned are the object of the process of the invention. It is part of the level of knowledge of the expert that certain results of separating steps are less useful than other combinations. For example the expert knows that when carrying out a preparative molecular sieve filtration after an analytical molecular sieve filtration, in addition to the unwieldy procedure, a poorer overall result with respect to the separating effect is also obtained than for the reverse sequence.

Molecular sieve filtration effects separation of the proteins according to their molecular weight. Since a predominant part of the concomitant foreign proteins has a different molecular weight to the bioactive RNP, their separation may be achieved in this manner. A hydrophilic molecular sieve which swells in water is used for separation of the substances according to their molecular weight. Examples of suitable molecular sieves are dextrans crosslinked using epichlorohydrin (sephadex), agaroses crosslinked using acrylamide (Ultrogels) and spatially crosslinked acrylamides (biogels), the exclusion limits of which are greater than the separation limits used for separation.

Molecular sieve filtration is preferably carried out as one of the first separating stages, if several separating stages are used. Depending on the length-diameter ratio of the columns used and the particle diameter of the gel matrix, which influence the theoretical number of plates of the column, molecular sieve filtration is designated as "preparative" or "analytical". It is designated as "preparative" if chromatography is carried out on columns having a dimensional ratio length:diameter up to 10:1 and a charge of up to 1/3 of the column content or with full utilisation of the total, matrix-typical separation volume. "Analytical" means an length-diameter ratio above 10:1, preferably about 50:1, and a charge up to a maximum 3 % of the column content, even for HPLC versions.

In preparative molecular sieve chromatography, gel matrices having as large as possible particle size are used to achieve rapid throughflow rates of the often somewhat viscous protein solutions at as low pressures as possible. In analytical molecular sieve filtration,

the particle size of the gel matrix is selected to be as small as possible to achieve a maximum theoretical number of plates for the column at technically and, in terms of safety, acceptable pressure and a flow rate for the mobile phase of 2 to 4 cm/hour. These parameters depend on the gel matrix structure and are different from gel to gel.

If several preparative molecular sieve filtrations are carried out one after another, the separation limit may be selected to be graded. Analytical molecular sieve filtration with correspondingly graded separation limits may be carried out following that. The exclusion limit of the gel used must in any case be greater than about 10,000 Dalton to facilitate volume distribution of the angiotropins between the stationary gel matrix phase and the mobile aqueous buffer phase.

The "exclusion limit" designates the hydrodynamic parameter of a dissolved particle, which corresponds to the pore size of the gel matrix. Particles having greater hydrodynamic parameter can no longer penetrate into the gel matrix (volume distribution coefficient $K_D = 0$). The "separation limit" designates a hydrodynamic parameter advantageously fixed to separate dissolved particles, and which lies between a volume distribution coefficient $K_D = 0$ and $K_D = 1$.

For molecular sieve filtration, the substances are dissolved in a liquid, which does not damage the substances, applied to the molecular sieve. A specific example of a suitable solvent is 0.003 mole/litre sodium potassium phosphate solution containing 0.3 mole/litre NaCl and 0.001 mole/litre cysteine and having a pH value of 7.4. After filtration, the fractions containing RNT are concentrated in the manner described below, and optionally subjected to a further purification step.

Suitable anion exchangers for purification of the substances are, for example dextran crosslinked using epichlorohydrin (sephadex) or cellulose matrices, to which functional groups having anion exchange capacity are coupled. They may be used again after use

due to regeneration. An equilibrated, weak anion exchanger pre-swollen in a buffer solution in the Cl form, such as DEAE-sephadex-A 50, is preferably used and the treatment is carried out at a pH value of 8 to 10. A specific example of such a buffer solution is 0.01 mole/litre tris HCl, which contains 0.04 mole/litre NaCl and 0.001 mole/litre cysteine and has a pH value of 8.0.

When using the anion exchanger, the substance fraction of such a quantity of anion exchanger is added, which is adequate for complete adsorption of the angiotropins and the positively adsorbed concomitant proteins. Two parts by volume of swollen anion exchangers conventionally suffice for this per volume of concentrated protein fraction. The reaction may be designed either as a chromatography process or as an easier to manage one-pot adsorption process. In the one-pot process, the supernatant liquid with the negatively adsorbed proteins is separated from the anion exchanger charged with the positively adsorbed RNP and other substances, for example by filtering (in the chromatography column), decanting or centrifuging (in the one-pot process). The charged anion exchanger is freed of adhering, negatively adsorbed compounds by washing with water or a salt solution, which has a maximum ion strength equivalent to 0.04 mole/litre NaCl, preferably at the most about 15°C. A specific example of a salt solution suitable for washing out is the tris HCl buffer solution mentioned of pH value 8.0.

The anion exchangers charged with RNP and other substances and freed of negatively adsorbed compounds is now eluted using an aqueous salt solution which does not damage proteins, and which has a corresponding ion strength greater than 0.04 mole/litre NaCl and a pH value between 4.0 and 10.0. A salt solution of high ion strength having a pH value of 5.0 to 7.0 is preferably used. A specific example of such a salt solution is a 2.0 mole/litre NaCl solution, which is buffered with 0.01 mole/litre piperazine HCl around pH value 6.5 and which contains 0.001 mole/litre cysteine.

If the anion exchange reaction is designed as a chromatography process, elution of the RNP and other substances may also be effected by a linear NaCl concentration gradient.

Suitable cation exchangers for purification of the protein fraction are, for example dextran crosslinked with epichlorohydrin (sephadex) or cellulose matrices, to which functional groups having cation exchange capacity are coupled. They may be used again after use due to regeneration. A weakly acidic cation exchanger in the Na^+ form, such as CM-sephadex C-50, is preferably used, and the treatment is carried out at a pH value of 4 to 6. The substance fractions may be diluted to facilitate adjustment of the charge equilibria before treatment with the cation exchanger using a salt solution which does not damage proteins, and which has a maximum ion strength equivalent to 0.04 mole NaCl/litre. It may serve at the same time for adjusting the pH value. A specific example of such a salt solution is a 0.001 mole/litre potassium phosphate acetate buffer solution containing 0.04 mole/litre NaCl and having a pH value of 4 to 6. This cation exchange reaction may be permitted both as a chromatography process and as a technically easily manageable one-pot process.

The cation exchanger is added to the substance fraction in a quantity which is adequate to adsorb the protein fraction. About 2 parts by volume of swollen ion exchanger per part by volume of protein fraction conventionally suffice therefor. The supernatant liquid is then separated off from the cation exchanger charged with the substances, for example by decanting or centrifuging. The charged cation exchanger is freed of adhering, non-adsorbed compound by washing with water or a salt solution, which has a maximum ion strength equivalent in 0.04 mole/litre NaCl, preferably at a pH value of about 4 to 6 and a temperature of preferably at most about 15°C. A specific example of a salt solution suitable for washing out is the potassium phosphate acetate buffer solution mentioned of pH value 5.0.

The cation exchangers charged with the substances and freed of negatively adsorbed compounds is now eluted using an aqueous salt solution which does not damage proteins and nucleic acids. A salt solution of high ion strength having a pH value of 4 to 10 is preferably used for this. Specific examples of such salt solutions are an aqueous 0.5 mole/litre potassium phosphate solution of pH value 6.5 to 7.5 or a 2 to 5 mole/litre sodium chloride solution of the same pH value.

For chromatography on hydroxylapatite, salts possibly present from preceding steps, for example ammonium sulphate and above all phosphates, are removed before application to the hydroxylapatite, preferably by dialysis or ultrafiltration on a membrane having an exclusion limit of 500 Dalton. Apart from the increase in viscosity due to foreign additives however, only the phosphate concentration of the protein solution is critical for succeeding in chromatography on hydroxylapatite. Elution of the substances takes place due to a potassium phosphate concentration gradient, which is preferably linear. The fractions containing RNP are collected and concentrated in the manner mentioned below.

The use of hydroxylapatite is of considerable importance for the structure-protecting pure recovery of RNP. However, it is associated with considerable difficulties, for technical and economic reasons, to chromatograph larger substance volumes on hydroxylapatite columns. On the one hand, hydroxylapatite tends namely to clog very severely for larger substance volumes and thus becomes unusable. On the other hand, hydroxylapatite is expensive, which stands in the way of its use on a larger scale. For these reasons, it is preferred in the process of the invention to separate off a large part of the concomitant foreign proteins by suitable process steps from the substance fractions, in which the bioactive RNP are present as traces, even before chromatography on hydroxylapatite, and thus to reduce decisively the protein volume, which has to be applied to the hydroxylapatite column.

In zone precipitation chromatography (see J. Porath, Nature, Volume 196 (1962), page 47-48), protein impurities of bioactive RNP are separated off by salting out fractionation of the proteins by means of a salt concentration gradient.

The basic principle of protein separation by means of zone precipitation chromatography is the different, structure-related reversible solubility properties of proteins. They belong to the most sensitive molecular separation parameters and have often been used as a criterion for the detection of molecular uniformity of a protein. Hence, temperatures and pH value, dimension of the column, type of salt, shape of the gradient and charge of the column may be varied within a relatively wide range.

The temperature for zone precipitation chromatography may be about 0 to 40°C. A temperature range from about 0 to 10°C, in particular about 4 to 6°C, is preferred. The pH value may lie between about 4 and 10; a pH value is preferably in the range from 6 to 8, in particular about 7. The ratio of length:diameter of the column used should be greater than about 10:1, a ratio of 30 to 100:1, in particular about 50:1, is preferred. Suitable salts are all salts with effect which do not damage proteins and nucleic acids. Examples of such salts are sodium potassium phosphate, ammonium sulphate and sodium sulphate. Ammonium sulphate is preferably used.

The salt concentration gradient may have any shape, as long as the salting-out points of the proteins are separated according to migration path. Linear concentration gradients, in particular a rising linear concentration gradient of 25 to 100 % ammonium sulphate saturation, are preferred. Charging of the column is at the most about 5 %, preferably about 1 %.

Circulation or cascade molecular sieve filtration may be carried out under the conditions which are described above for analytical molecular sieve filtration. The same molecular sieves and the same column conditions may be used. Sephadex G 50 is preferred for a

length-diameter ratio for the column of at least about 50:1 and a charge at the most of about 3 % of the column content. The solvents used in analytical molecular sieve filtration are preferably used as solvent and for elution.

In circulation molecular sieve filtration, the eluate is recirculated into the same column at the fixed separation limits. The migration path of the proteins is extended differentially in this manner. In a different embodiment, cascade molecular sieve filtration, the eluate is passed to a new column with the same or similarly defined parameters at the fixed separation limits.

The substance solutions obtained containing bioactive RNP may be purified of undesirable salts and concentrated to give subsequent fractionations of the proteins between the purification steps illustrated above. This concentration (separation of the large part of the aqueous salt solution from the proteins) may be effected in a different manner. For example, the bioactive RNP and the concomitant substances may be concentrated by ultrafiltration or water-removing dialysis on a membrane with the exclusion limit 500 Dalton or by lyophilisation. Molecular sieve filtration may thus also be used modified in conventional manner by selecting the appropriate mobile phase. For molecular sieve filtration, about 0.4 mole/litre ammonium sulphate is preferably added to the substance solution. In contrast to higher concentrations, the ammonium sulphate at this concentration has a strong salting-down effect with respect to proteins. As a result of these measures, the proteins are accordingly kept in solution during molecular sieve filtration. Furthermore, ammonium sulphate prevents bacterial growth and inhibits certain enzymes. Hence it contributes to stabilisation of the bioactive RNP, particularly if chromatography is carried out at higher temperatures (above about 20°) and under non-sterile conditions.

The temperature and pH conditions are not particularly critical when carrying out the purification steps. If maintaining the native conformation of the substances is intended,

maintenance of a temperature in a range from about 0 to 8°C, preferably about 0 to 4°C, is advisable. Furthermore, the separation and purification stages have to be carried out under essentially physiological pH and salt conditions. A considerable advantage of the process of the invention consists in that the maintenance of these conditions is easily possible for the first time. The substance solution is preferably also treated with about 0.001 mole/litre cysteine to prevent oxidation.

The bioactive RNP obtained may be stored in a buffered physiological salt solution, for example in 0.0015 mole/litre sodium potassium phosphate solution containing 0.15 mole/ (0.9%) NaCl and 0.001 mole/litre cysteine and having a pH value of 7.4, after conventional filter sterilisation (0.2 µm pore width), naturally and biologically active even at room temperature (for at least 200 hours) or frozen at -25°C (for at least 5 years). This stability of the bioactive RNP may be regarded, inter alia, as one of the criteria for its highly pure state.

The RNP of the invention may also be produced using chemically or biologically synthesised partial sequences or parts and homologous sequences thereof. It is preferable if the chemically or biologically synthesised oligonucleotides or anti-sense nucleotide sequences in vivo or in vitro, which code partial sequences given according to claim 1, having at least 6 bases are used in the PCR reaction, or anti-sense bioprocess technology is used.

The examples illustrate the invention. The examples describe the recovery of RNP morphogens starting from leucocytes from porcine blood. However, the invention is not restricted to this embodiment. Cells from the reticulo-endothelial system or inflammation tissue, wound tissue or fluid (exudate) of other mammals may also be used.

A further object of the present invention is an antibody directed against an above protein or fusion protein or a partial sequence thereof. The antibodies may be monoclonal,

polyclonal or synthetic antibodies or fragments thereof, for example Fab, Fv or soFv fragments. They are preferably monoclonal antibodies. For production it is favourable to immunise animals, in particular rabbits or chickens for polyclonal antibodies and mice for monoclonal antibodies, with an above (fusion) protein or fragments thereof. Further "boosters" may be given to the animals using the same (fusion) protein or fragments thereof. The polyclonal antibody may then be obtained from the serum or egg yolk of the animals. The antibodies of the invention may be produced according to standard processes, wherein the protein coded by the nucleic acid molecules of the invention or a synthetic fragment thereof serve as immunogen.

Monoclonal antibodies may be produced, for example by the process described by Köhler and Milstein (Nature 256 (1975), 495) and Galfré, Meth. Enzymol. 73 (1981), 3, wherein mice myeloma cells are fused with spleen cells originating from immunised mammals. These antibodies may be used, for example for immunoprecipitation of the RNPs discussed above or for isolating related structures. The antibodies may be bound, for example in immunoassays in liquid phase or to a solid carrier. The antibodies may thus be marked in different ways. Suitable markers and marking processes are known in the expert field. Examples of immunoassays are ELISA and RIA.

The present invention also relates to the use of the RNPs described above and/or antibodies as medicaments. These medicaments optionally additionally contain a pharmaceutically acceptable excipient. Suitable excipients and the formulation of such medicaments are known to the expert. Suitable excipients include, for example phosphate-buffered saline solutions, water, emulsions, for example oil/water emulsions, wetting agents, sterile solutions etc. The administration of the medicaments may be effected orally or parenterally. The processes for parenteral administration include the topical, intra-arterial (for example directly to the tumour), intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal or intranasal administration. Suitable dose is determined by the treating physician and depends on

various factors, for example on the age, the sex, the weight of the patient, the type of administration etc. In a preferred embodiment, the RNPs of the invention may be given individually or as a mixture locally for mammals, for example humans, in a quantity of > 1 fmole. The threshold dose of efficacy in vivo is > 50 fmoles, preferably 2.5 fmoles. These medicaments are suitable for specifically influencing angiomorphogenesis and the vascular state of a tissue of a body of a mammal. These medicaments may also contain at least one anti-RNP immunoglobulin and/or molecular-biological equivalent structures to fulfil the same tasks. The resulting medicament is preferably used for functional influencing of angiogenesis.

The invention is further described using the figures, which show:

Figure 1: Energy-minimised secondary structure of ARNA I

Figure 2: Energy-minimised secondary structure of ARNA VI

AA34

Amended claims as enclosed to the International Preliminary Examination report (2 pages in total)

FRAUNHOFER-GESELLSCHAFT

New patent claims

1. Metal-containing ribonucleotide proteins containing a protein from the family of S100 proteins, an RNA and copper as metal ion in the form of a ternary complex, characterised in that the RNA of the ternary complex has the following sequences:

(a1) (ARNA I)

Klon-3a (ARNA I)
AAAAAAAAAGGUUUUCAUGCGUGCUCACAGAUCAGCUCUUUCUGGAUUGAAAAGCU
AAGCACAGAACAUGGGAAAAUUCCUUUCAUAUGGCUGUGUUUACAAACAAAAAGU
AUAAACAUCUUGAGCAAACAGAAAUGGUGAGGAAAACUUUGUUAGCAGAUUAG

or

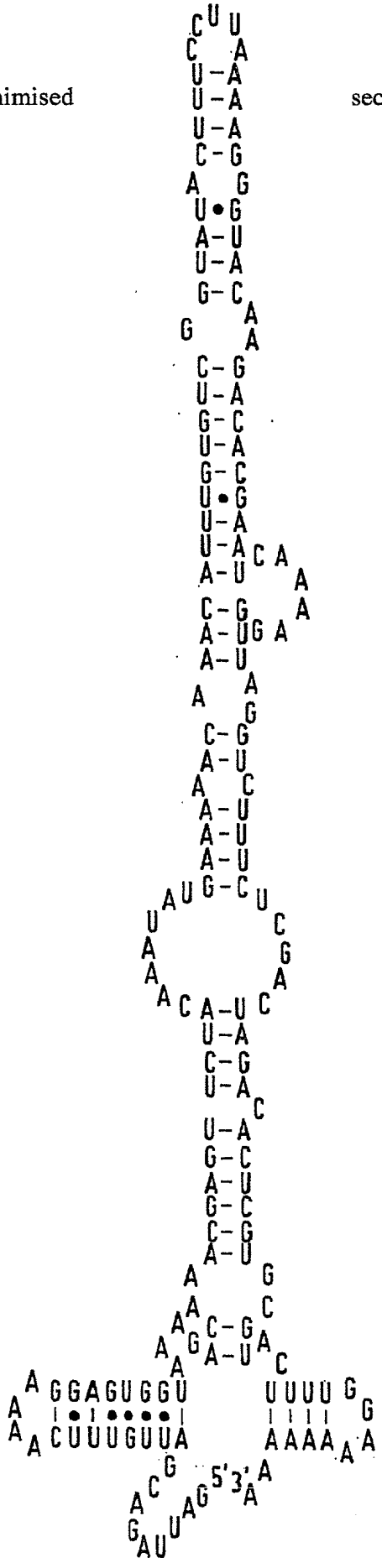
(a2) ARNA VI

Klon-P10 (ARNA VI)
UUACAGCUCUUCUGUUUAUAAGUUAUUCAAUACCAAAUUAGUAGUUUGUAUGUUA
UAAAUUUGUAGGAAAAUAAUUAUAUAUGCUUACUUUGUACAUAAAAAUAAAAACAU
GACUUCUUUAGACACUCCUUCAUUAGAAAUAAAAUAAAAUAAACUAUUAGCAGUUU
GACUUCAUGUUCUGUCUGUAGGUCAUGGAAUCCUGUCCUUACAAUAUUUAUUGAU
UGUGAAAAUAUCAGUAAAUAAGCAAUUGAAUAUGUUUACCUUUUCUUCUAGUCAC
UAUGUUCUUAGAGUUAUGACA

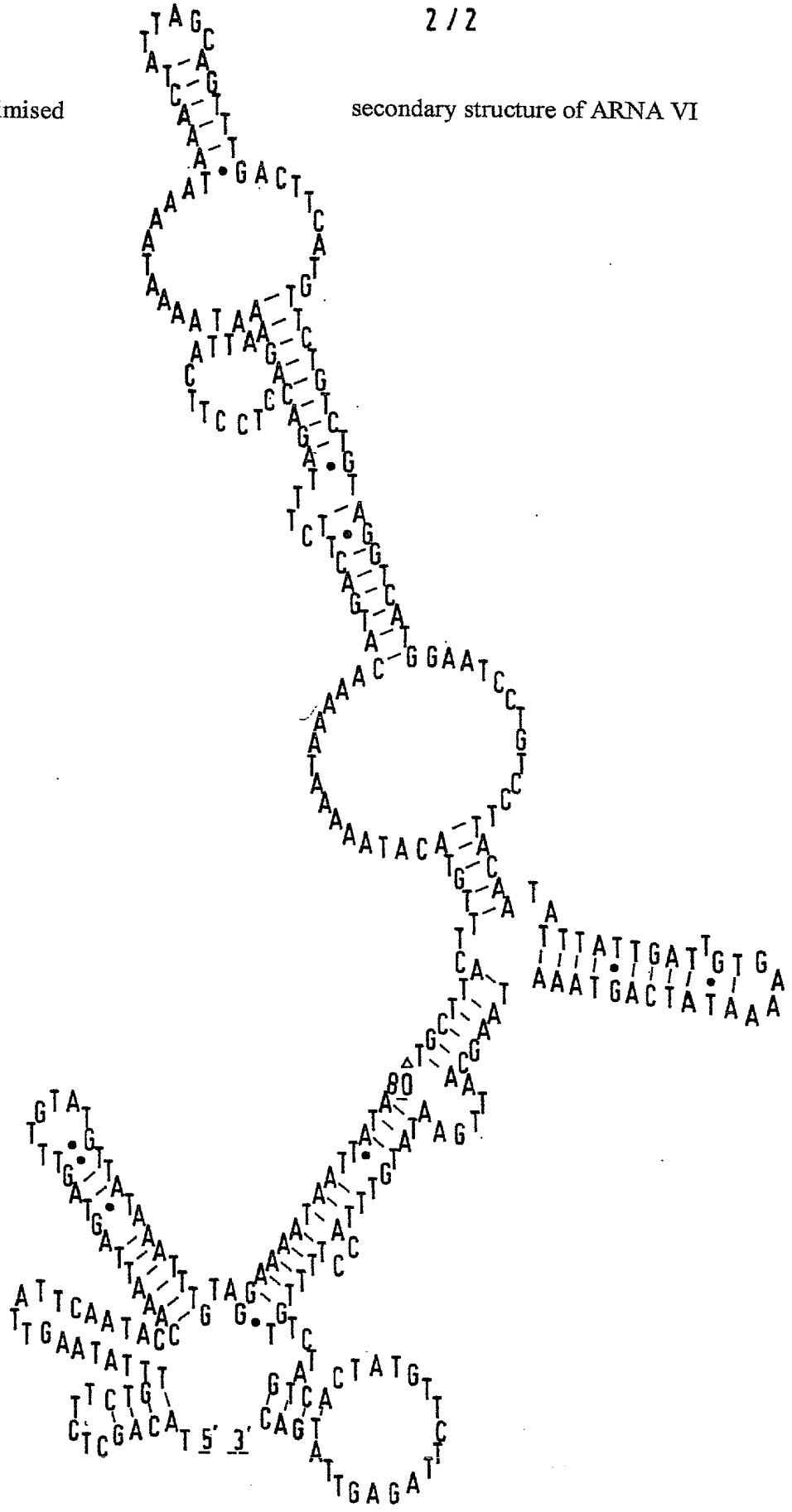
2. Process for producing a metal-containing ribonucleotide protein according to claim 1, characterised in that leucocytes or inflammation tissue is homogenised or leucocytes are cultivated and the resulting RNPs are recovered from the homogenates or from the supernatants of the culture solution by standard methods.

3. Use of the ribonucleotide proteins according to claim 1 and/or molecular-biological equivalent structures and/or fragments and/or derivatives for producing a medicament for specifically influencing angiogenesis.

Energy-minimised secondary structure of ARNA I



Energy-minimised secondary structure of ARNA VI



In re Appln. of Kiesewetter et al.
Attorney Docket No. 206579

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1-10

Full name of **sole or first inventor:** Stefan KIESEWETTER

Inventor's signature _____

Date November 10, 2000

Country of Citizenship: Germany

Residence: Osterfeldern, Germany (DE)
(city/state or country)

Post Office Address: Wagenmannsteige 5, D-73760 Osterfeldern, Germany
(complete mailing address)

2-10 Full name of **second joint inventor,** if any: Eckehard KUHN

Inventor's signature _____

Date _____

Country of Citizenship: Germany

Residence: Frickenhausen, Germany (DE)
(city/state or country)

Post Office Address: Im Dorf 14, D-72636 Frickenhausen, Germany
(complete mailing address)

Full name of **third joint inventor**, if any: Bridgitte KOCH-PELSTER

Inventor's signature _____

Date _____ Country of Citizenship: Germany

Residence: Backnang, Germany
(city/state or country)

Post Office Address: Ludwigsburger Strasse 27, D-71522 Backnang, Germany
(complete mailing address)

Full name of **fourth joint inventor**, if any: Herwig BRUNNER

Inventor's signature _____

Date _____ Country of Citizenship: Germany

Residence: Stuttgart, Germany
(city/state or country)

Post Office Address: An der Betteleiche 6, D-70569 Stuttgart. Germany
(complete mailing address)

PATENT
Attorney's Docket No. 206579

COMBINED DECLARATION AND POWER OF ATTORNEY

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☒ original ☐ design ☐ supplemental
☐ national stage of PCT
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METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

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PRIOR FOREIGN PATENT, UTILITY MODEL, AND DESIGN REGISTRATION APPLICATIONS						
COUNTRY	APPLICATION	DATE OF FILING (day,**month**,year)	PRIORITY CLAIMED UNDER 35 U.S.C. § 119			
Germany	198 11 047.2	13 March 1998	X	YES		NO
				YES		NO
				YES		NO

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U.S. APPLICATIONS			Status *(check one)*		
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1. 0 /					
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4. PCT/EP98/07722	30 Nov. 1998		X		
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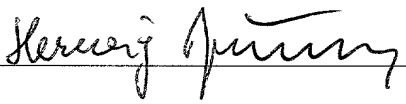
Inventor's signature 

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97/33267 97b2267

PATENT
Attorney's Docket No. 206579

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Date _____ Country of Citizenship: Germany

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Post Office Address: Im Dorf 14, D-72636 Frickenhausen, Germany
(complete mailing address)

Full name of **third joint inventor,** if any: Brigitte KOCH-PELSTER

Inventor's signature Brigitte Koch-Pelster

Date 25.10.2000 Country of Citizenship: Germany

Residence: Backnang, Germany
(city/state or country)

Post Office Address: Ludwigsburger Strasse 27, D-71522 Backnang, Germany
(complete mailing address)

Full name of **fourth joint inventor,** if any: Herwig BRUNNER

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Post Office Address: Wagenmannsteige 5, D-73760 Osterfeldern, Germany
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Full name of **second joint inventor,** if any: Eckehard KUHN

Inventor's signature Eckehard Kuhn

Date 10. November 2000 Country of Citizenship: Germany

Residence: Frickenhausen, Germany
(city/state or country)

Post Office Address: Im Dorf 14, D-72636 Frickenhausen, Germany
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PCT APPLICATION NO.	PCT FILING DATE (day,**month**,year)	U.S. APPLN. NOS. ASSIGNED (if any)	PUBLISHED	PENDING	ABANDONED
4. PCT/EP98/07722	30 Nov. 1998		X		
5.					
6.					

DETAILS OF FOREIGN APPLICATIONS FROM WHICH PRIORITY CLAIMED UNDER 35 U.S.C. §119 FOR ABOVE LISTED U.S./PCT APPLICATIONS				
ABOVE APPLN. NO.	COUNTRY	APPLICATION NO.	DATE OF FILING (day,**month**,year)	DATE OF ISSUE (day,**month**,year)
1.				
2.				
3.				
4. PCT/EP98/07722	Germany	198 11 047.2	13 March 1998	
5.				
6.				

SEQUENCE LISTING

<110> Kiesewetter, Stefan

Kuhn, Eckehard

Koch-Pelster, Brigitte

Brunner, Herwig

<120> METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES

<130> 206579

<140> 09/646,651
<141> 2000-09-13

<150> PCT/EP98/07722
<151> 1998-11-30

<150> DE 198 11 047.2
<151> 1998-03-13

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<170> PatentIn version 3.0

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Asp Gln Gly Thr Ile Asp Lys Ile Phe Tyr Asn Leu Asp Ala Asn Gln
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agacacuccu ucauuagaaa uaaaauaaaa uaaacuauua gcaguuugac uucauguucu 180

gucuguaggu cauggaaucc uguccuuaca auauuuauug auugugaaaa uaucaguaaa 240

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PCT09

RAW SEQUENCE LISTING DATE: 08/03/2001
PATENT APPLICATION: US/09/646,651A TIME: 13:56:33

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Kuhn, Eckehard
Koch-Pelster, Brigitte
Brunner, Herwig
<120> TITLE OF INVENTION: METAL-CONTAINING RIBONUCLEOTIDE POLYPEPTIDES
<130> FILE REFERENCE: 206579
<140> CURRENT APPLICATION NUMBER: 09/646,651A
<141> CURRENT FILING DATE: 2000-09-13
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<151> PRIOR FILING DATE: 1998-11-30
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<151> PRIOR FILING DATE: 1998-03-13
<160> NUMBER OF SEQ ID NOS: 4
<170> SOFTWARE: PatentIn version 3.0
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Tyr Ser Val Arg Leu Gly His Tyr Asp Thr Leu Ile Lys Arg Glu Leu
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Lys Tyr Leu Ile Thr Lys Glu Leu Pro Asn Thr Leu Lys Asn Thr Lys
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Asp Gln Gly Thr Ile Asp Lys Ile Phe Tyr Asn Leu Asp Ala Asn Gln
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PATENT APPLICATION: **US/09/646,651A** TIME: 13:56:33

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VERIFICATION SUMMARY DATE: 08/03/2001
PATENT APPLICATION: **US/09/646,651A** TIME: 13:56:34

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